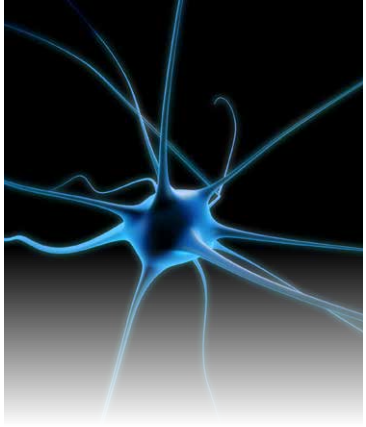
Application Note

Neuroscience/Clinical



Aminothiols and Disulfides

- **Universal method for aminothiols, disulfides and thioethers**
- **No derivatization step required**
- **Single flow cell with diamond working electrode**
- **Easy and sensitive analysis**
- **Elution & detection of all compounds within 10 min**

Introduction

Aminothiols (Cysteine, Cysteinylglycine, Glutathione and Homocysteine,) and their disulfides (Cystine, oxidized Glutathione and Homocystine) serve numerous vital functions in biochemistry, including detoxification and regulation of cellular metabolism, enzymatic activity, and protein trafficking. Glutathione (GSH), a tripeptide composed of glutamate, cysteine (Cys) and glycine (Gly), has an important role as an antioxidant in detoxification of harmful substances and xenobiotics [1]. Homocysteine (Hcys) is considered as a biomarker for some cardiovascular, neurological and congenital diseases. Methionine (Met) and Cysteine are proteinogenic amino acids essential for the synthesis of proteins. Homocysteine, Methionine and GSH are metabolically related in a pathway including Cysteine and Cysteinylglycine (CysGly) as intermediates. For these reasons, determination of these aminothiols and disulfides is of great importance in the diagnosis of several diseases.

Although a variety of techniques are available to measure aminothiol concentrations in biological samples, high performance liquid chromatography with fluorescence detection (HPLC/FD) is still the most widely used [2]. However, fluorescence detection relies on the presence of a chromophore and aminothiols cannot be detected directly without prior derivatization with a suitable fluorescence label like for example 4-Fluoro-7-Sulfobenzofurazan (SBD-F). Moreover, in case

of the fluorescence detection of the disulfides one needs an additional chemical reduction step (for example using tributylphosphine) prior to derivatization to reduce them into thiols (SBD-F reacts with the sulfhydryl group only).

UHPLC in combination with electrochemical detection (UHPLC/ECD) offers a better alternative to fluorescence detection, because it allows the direct and sensitive detection in DC mode of all sulfur-containing compounds (thiols, disulfides and thioethers) without derivatization, using a single amperometric flow cell based on an a conductive diamond working electrode [3]. Conductive diamond has several advantages over conventional electrode materials such as a wide potential window in aqueous solutions, excellent chemical inertness and long-term response stability.

Summary

In this application note the easy and sensitive analysis of aminothiols, disulfides and thioethers is demonstrated using an ALEXYS UHPLC/ECD analyzer using the DECADE Elite electrochemical detector (Figure 1). The method itself is based on separation using ion-pairing reverse phase LC followed by direct detection in DC mode of all sulfur-containing analytes without derivatization, using an amperometric flow cell equipped with a Magic Diamond™ (MD) working electrode. A mix of 9 biologically relevant aminothiol and disulfide standards was analyzed. To demonstrate the applicability of this method for the analysis of sulfur-containing compounds in body fluids, a whole blood sample was analyzed and the levels of amino thiols and disulfides quantified.

Figure 1: ALEXYS UHPLC/ECD Analyzer for Aminothiols and Disulfides.

Method

The ALEXYS Neurotransmitter base platform, single channel (p/n 180.0091E) consisting of the DECADE Elite detector, LC110S pump and AS 110 UHPLC autosampler was used for the method development. The results presented in this application note were obtained using the UHPLC/ECD conditions as specified in table 1 unless stated otherwise.

Table 1

UHPLC/ECD Conditions	
HPLC	ALEXYS Neurotransmitter base platform, single channel with DECADE Elite detector, LC110S pump and AS 110 UHPLC autosampler (optional)
Column	Acquity UPLC BEH C18, 100 x 1mm ID, 1.7µm
Pre-column filter	Acquity UPLC column in-line filter kit + 6 frits (0.2 µm)
Mobile phase	100mM Phosphoric Acid, 100mM Citric Acid , 500 mg/L Octanesulphonic acid , pH = 3.0
Flow rate	100 µL/min
Temperature	45°C (separation and detection)
$V_{injection}$	1 µL (Partial Loop fill with 5 µL loop)
Flow cell	FlexCell with Magic Diamond (MD) electrode and Salt-Bridge Ag/AgCl reference electrode, spacer 50 µm
E cell	+ 1800 mV vs. Ag/AgCl
Range	2 µA/V and 50 µA/V
ADF™	Off
I-cell	about 1 µA

Separation

An Acquity UPLC BEH C18 column (100 x 1mm ID, 1.7µm) was used for the separation of the thiols and disulfides. The compounds are isocratically eluted under acidic conditions (pH 3.0) using a citrate-phosphate buffer with 500 mg/L 1-Octanesulphonic acid (ion-pairing agent) as a mobile phase. Separation of amino acids is affected by pH, concentration ion-pairing reagent, percentage modifier and temperature. Decreasing the mobile phase pH below 4 which is the pKa of carboxyl group, will result in an increase of retention times.

Amino acids with a free amine group are protonated at acidic pH. A citrate-phosphate buffer has a wide pH range (pH 2.6 – 6.2), which offers the possibility to tune the elution behavior by adjusting the pH of the mobile phase if necessary. The mobile phase was 100% aqueous and no organic modifier was used to assure that the fast eluting compounds (CSSC and CSH) could be separated with sufficient resolution for reliable quantification. A column temperature of 45°C was found to be the optimal temperature for separation and detection. At 45°C all 9 compound peaks were baseline separated (resolution R >1.8) and eluting within 8 minutes at a flow rate of 100 µL/min. An example chromatogram obtained with a 1 µL injection of a 10 µmol/L mix of 9 standards dissolved in mobile phase is shown in figure 3.

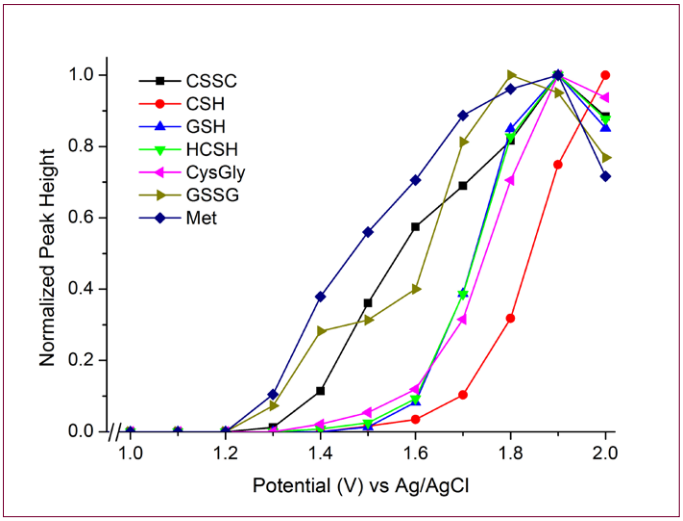


Figure 2: Voltammograms of 10 µmol/L standards using potentials between 1 and 2 V.

Detection

For the detection of the sulfur-containing compounds a FlexCell was used equipped with a diamond working electrode (WE), Ag/AgCl salt-bridge reference electrode (RE) and graphitized teflon auxiliary electrode (AE). Diamond, a relatively new carbon-based electrode material, has the advantage that it is more inert (with respect to adsorption) and can be used over a much larger potential window compared to the more conventional glassy carbon electrode. The possibility to apply higher oxidation potentials without the problem of mobile phase oxidation (and hydrolysis) and adsorption of contaminants allows the direct and stabile detection of thiols and disulfides in DC mode. A hydrodynamic voltammogram was recorded to determine the optimal oxidation potential for detection. Therefore, a series of chromatograms were recorded with different potential settings in the range of 1000 to 2000 mV with 100 mV steps. In figure 2 the obtained voltammograms (Normalized peak height as a function of applied potential) are shown for 7 of the amino thiol and disulfide standards. A potential of 1800 mV versus Ag/AgCl was chosen as the optimal potential for the detection of the sulfur-containing compounds. Note that at this potential not all compounds are at their maximum response, but that the choice is based on the assessment of both signal and background noise (at 1800 mV the S/N ratio is the highest).

Sample preparation

Whole blood: A small amount of blood obtained by dermal puncture (finger) was collected in a 1.5 mL Eppendorf vial. Protein precipitation was achieved by pipetting 10 µL of the collected whole blood into an Eppendorp vial containing 300 µL 0.5 mol/L perchloric acid (PCA), which was subsequently vortexed for 1 minute followed by centrifugation for 15 minutes at 6000 RPM (2000 x g). The clear supernatant above the sediment was pipetted into a 200 µL TPX micro vial (with glass insert) placed in the autosampler at 4°C and immediately analyzed.

Results

An example chromatogram obtained with a 1 µL injection of a 10 µmol/L mix of 9 standards dissolved in mobile phase is shown in figure 3.

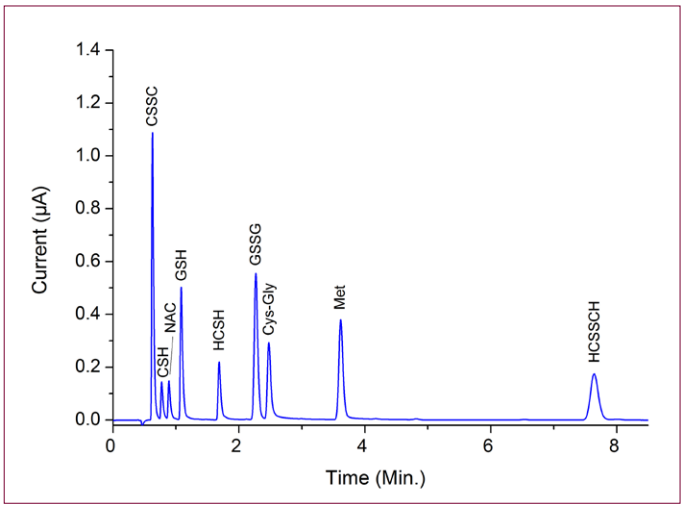


Figure 3: Chromatogram of a 1 µL injection of 10 µmol/L standard mix diluted in mobile phase: Cystine (CSSC), Cysteine (CSH), N-acetyl Cysteine (NAC), Glutathione (GSH), Homocysteine (HCSH), L-Cysteine-L-Glycine (Cys-Gly), oxidized Glutathione (GSSG), Methionine (Met) and Homocistine (HCSSCH).

It is evident from figure 3 that all 9 aminothiols and disulfides are eluting within 8 minutes and all peaks are baseline separated (resolution ≥ 1.8) with peak efficiencies in the range of 20,000 - 160,000 theoretical plates/meter. The tailing factor of all component peaks were smaller than 2.0. GSH and GSSG eluted at 1.1 and 2.3 minutes, respectively. This demonstrates the suitability of this method for the quantification of aminothiols and disulfides in biological samples. Examples of such bioanalysis are shown in the next section of this application note.

Linearity, Repeatability & LOD

Linearity

Calibration curves were recorded in the biologically relevant concentration ranges of 0.2 – 10 µmol/L and 10 – 100 µmol/L using calibration standards based on a mix of the 9 thiols and disulfides dissolved in mobile phase. The method showed a good linear detector response with correlation coefficients for the calibration curves better than 0.999 and 0.997 (respectively) for all compounds. The calibration curves in the concentration range of 0.2 – 10 µmol/L are shown in figure 4 as an example.

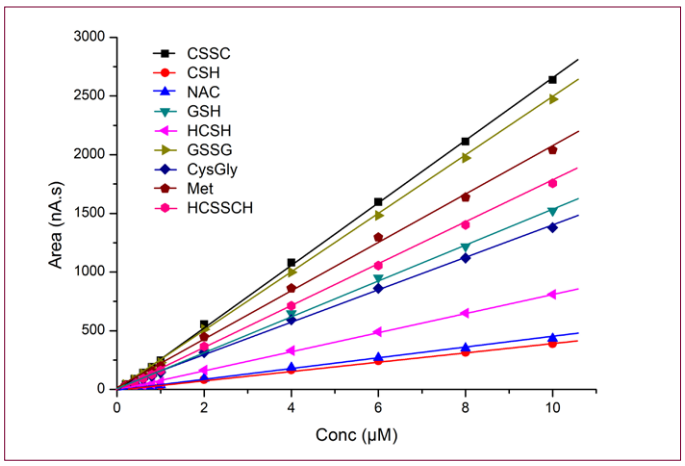


Figure 4: Calibration curves for all 9 aminothiols and disulfides dissolved in mobile phase (concentration range 0.2 – 10 µmol/L).

To determine the dynamic range for GSH and GSSG the linearity was evaluated at even higher concentrations up to 1 mmol/L. In figure 5 the calibration plots for both compounds are shown.

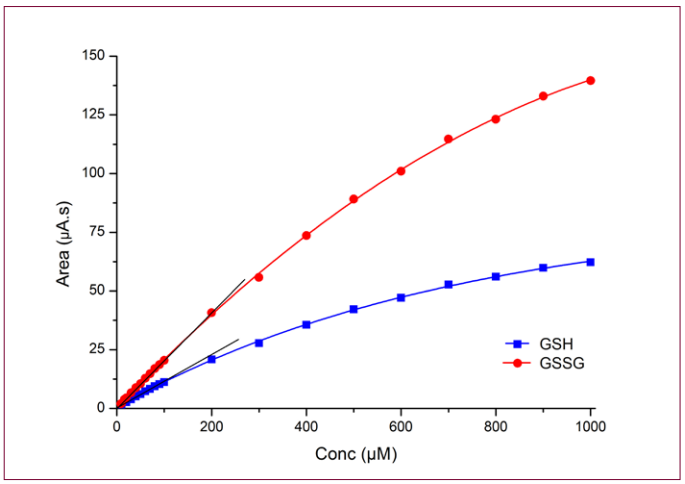


Figure 5: Calibration curves for reduced (GSH) and oxidized Glutathione (GSSG) in the concentration range 10 – 1000 µmol/L.

It is evident from figure 5 that the Limit of Linearity (LOL) of the detector response for GSH is about 100 µmol/L and for GSSG is about 200 µmol/L. Note that the linear range of the method can be extended by increasing the cell volume of the electrochemical flow cell. This can be simply achieved by increasing the spacer thickness (a spacer with a thickness of 50 µm was used in this study).

Repeatability

The repeatability of the method was evaluated by executing 10 repetitive injections (1 µL injection volume) of a 1 and 10 µmol/L standard mix dissolved in mobile phase. The relative standard deviations (RSD%) for retention time, peak area and height are shown in table 2.

Table 2

Repeatability 1 µL injections (n=10) of a 1 and 10 µmol/L standard mix in mobile phase

Component	*1 µmol/L standard mix*			*10 µmol/L standard mix*		
	RSD tR (%)	*RSD Height (%)*	*RSD Area (%)*	*RSD tR (%)*	*RSD Height (%)*	*RSD Area (%)*
Cystine (CSSC)	0.4	0.5	0.6	0.2	1.7	1.6
Cysteine (CSH)	0.3	1. 5	1.3	0.2	1.8	1.8
N-acetyl Cysteine (NAC)	0.2	1.0	0.8	0.2	1.6	1.7
Glutathione (GSH)	0.2	0.9	0.8	0.1	1.6	1.7
Homocysteine (HCSH)	0.1	0.9	0.9	0.1	1.7	1. 7
Glutathione oxidized (GSSG)	0.1	0.4	0.5	0.1	1.7	1.7
Cysteinylglycine (CysGly)	0.1	0.8	2.0	0.1	1.7	1.6
Methionine (Met)	0.1	0.5	0.8	0.1	1.7	1.7
Homocistine (HCSSCH)	0.1	1.6	2.4	0.1	1.7	1.7

The RSD's were ≤0.4% or lower for retention time, and ≤ 2% for both peak area and height for all thiols and disulfides. The long-term repeatability was assessed with a standard solution containing a high concentration GSH and GSSG of 50 µmol/L dissolved in mobile phase.

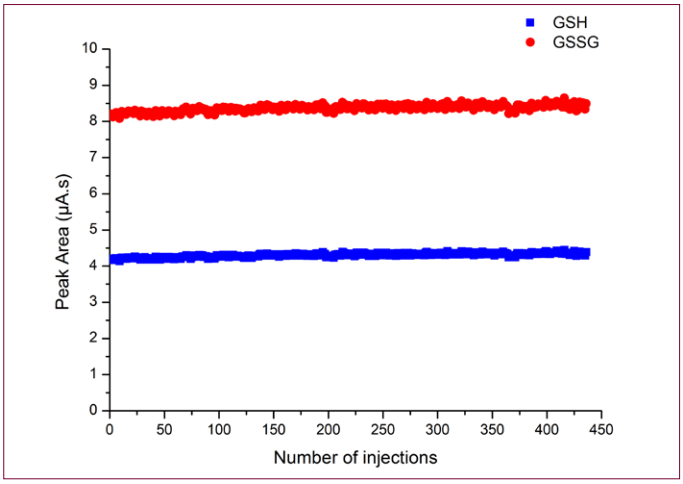


Figure 6: Long-term repeatability of DC amperometric detection of reduced (GSH) and oxidized (GSSG) Glutathione on a diamond working electrode. Sample concentration 50 µmol/L, injection volume 1 µL.

The relative standard deviation (RSD%) for the peak area for 450 injections was better than 1.5% for both GSH and GSSG. This demonstrates the excellent response stability of the diamond electrode material even at high analyte concentrations.

Limit of Detection (LOD)

The Limit of Detection (LOD) for all thiols and disulfides are listed in table 3. The LOD's are calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 30 segments of 0.5 min). In Figure 7 an example chromatogram is shown of a 1 µL injection of a concentration near the LOD (50 nmol/L) of the standard mix in 0.5 mol/L PCA.

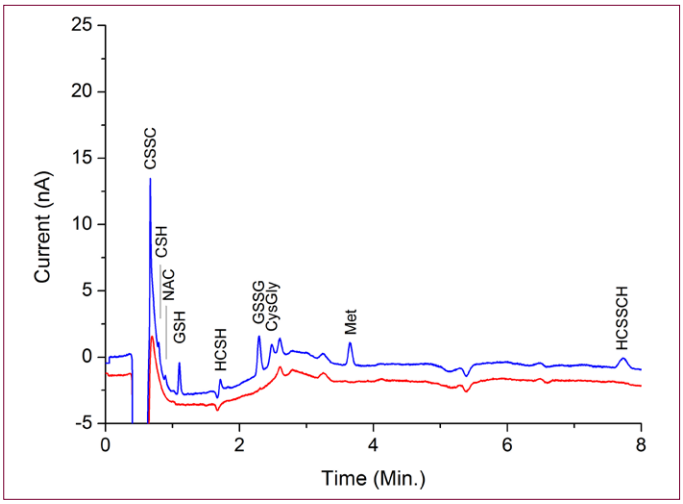


Figure 7: Overlay of a blank (red trace, 0.5 mol/L PCA) and a chromatogram of a 1 µL injection of 50 nmol/L standard mix diluted in 0.5 mol/L PCA (blue trace): Cystine (CSSC), Cysteine (CSH), N-acetyl Cysteine (NAC), Glutathione (GSH), Homocysteine (HCSH), L-Cysteine-L-Glycine (Cys-Gly), oxidized Glutathione (GSSG), Methionine (Met) and Homocistine (HCSSCH).

Table 3

Limit of Detection (LOD), S/N=3				
Component	*CLOD*		*On-column*	
	nmol/L	*ng/mL*	*fmol*	*pg*
Cystine (CSSC)	4	1.0	4	1.0
Cysteine (CSH)	40	4.8	40	4.8
N-acetyl Cysteine (NAC)	35	5.7	35	5.7
Glutathione (GSH)	12	3.7	12	3.7
Homocysteine (HCSH)	36	4.9	36	4.9
Glutathione oxidized (GSSG)	8	4.9	8	4.9
Cysteinylglycine (CysGly)	20	3.6	20	3.6
Methionine (Met)	11	1.6	11	1.6
Homocistine (HCSSCH)	25	6.7	25	6.7

A detection limit of < 10 pg on-column was achieved for all compounds. For GSH and GSSH the detection limit was even below 5 pg on-column, which corresponds to a molar concentration detection limit of about 10 nmol/L when using an injection volume of only 1µL.

Sample Analysis

In figure 8 an example chromatogram obtained with a whole blood sample is shown. Note that only the relevant time window (first 4 minutes) of the chromatogram is shown were the analytes of interest are eluting. The actual run duration was 20 minutes due to the presence of late eluting components in the blood sample. The sample preparation of the whole blood sample was fast and simple and consisted of a protein precipitation step followed by centrifugation as described in the method section. The deproteinized blood samples were analyzed immediately after sample preparation to assure minimal sample degradation/oxidation of the aminothiols. It should be stated that the sample preparation procedure described is not validated and is only used to obtain a series of example chromatograms.

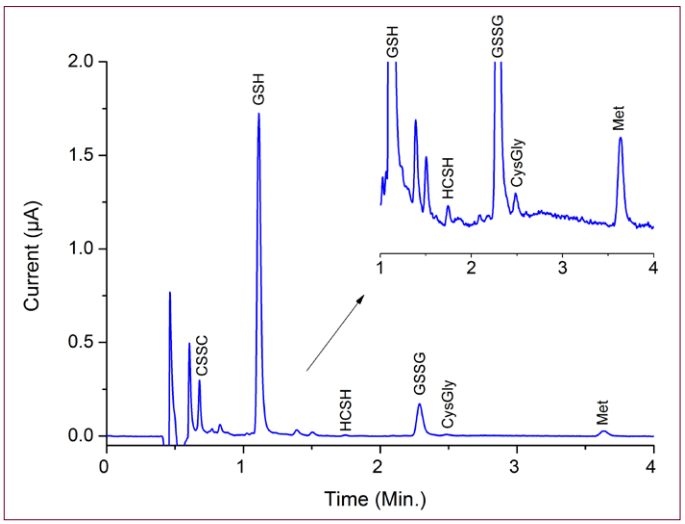


Figure 8: Chromatogram obtained from a 1 µL injection of a whole blood sample. Top-right inset: zoom-in on response of HCSH, CysGly and Met.

The concentration of free thiols and disulfides in the blood sample were calculated using the standard mix dissolved in 0.5 mol/L PCA, see table 4. The quantified levels of free Glutathione (GSH and GSSG) in the whole blood sample are within the concentration range reported in literature [4-6]. Besides Glutathione also Homocysteine, Cysteinylglycine, Methionine and Cystine could be quantified.

Table 4

Chromatogram obtained from a 1 µL injection of a whole blood sample. Top-right inset: zoom-in on response of HCSH, CysGly and Met.	
Component	*Concentration (µmol/L)*
Cystine (CSSC)	38.1
Glutathione (GSH)	872.1
Homocysteine (HCSH)	6.3
Glutathion oxidized (GSSG)	76.8
Cysteinylglycine (CysGly)	4.8
Methionine (Met)	18.9

Conclusion

The ALEXYS UHPLC/ECD Analyzer with the aminothiols kit offers a tailored solution for the analysis of both aminothiols and disulfides. The method is based on EC detection in DC mode of all sulfur-containing analytes without derivatization, using an amperometric flow cell with diamond working electrode.

References

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Ordering number

180.0091E	ALEXYS UHPLC/ECD BP, 1 Ch
180.0509	ALEXYS Aminothiols SCC kit, MD
191.0035UL	AS 110 autosampler UHPLC cool 6p, LAN
250.1163	Acquity UPLC BEH C18, 1.7µm, 1 x 100mm
250.1166	UPLC in-line filter kit + 6 frits